Remarks/Arguments

Withdrawal of the previous rejection under 35 USC § 102(b) over Riedel, and the previous rejections under 35 U.S.C. § 112, 1st paragraph are respectfully acknowledged.

Claims 33-34, 36-37, 43-50, 59-65, 71-78, and 87-89 are pending in the application and stand rejected. Claims 33, 43, 59, 62, 63, 71, and 87-89 are amended. Claims 90-120 are new.

The claimed invention is fully supported by instant specification and by the original specification filed as U.S. Ser. No. 07/154,206, filed Feb. 10, 1988, and issued as U.S. Patent No. 4,980,281 (the "'281 patent"). New claims 90 to 120 are directed to a method of determining whether a chemical agent specifically inhibits or activates a particular enzyme in a cell. Support is found in the Specification, for example, at page 1, lines 15-18 ('281 patent, Col. 1, lines 10-12), page 3, line 31 to page 4, line 4 ('281 patent, Col. 2, lines 25-33), and page 21, lines 1-6 ('281 patent, Col. 9, lines 25-35). Support for Claim 120 is found, for example, at page 30, lines 22-25 ('281 patent, Col. 14, lines 2-4). Claims 87-89 have been amended to correct a typographical error. Claims 33, 43, 59, 63, and 71 have been amended so as to relate the phenotypic response observed in the cells of the test method to the determination of whether a tested chemical agent is a direct inhibitor or activator of the enzyme of interest (see below). The amendment should not be considered a narrowing amendment with respect to the doctrine of equivalents, because it does not narrow or change in any way the test method that is claimed. The amendment merely states more clearly the connection between the result obtained by carrying out the method steps, and the determination being sought.

Rejection Under 35 U.S.C. § 102

Claims 33-34, 36, 43-44, 46, 47, 49 and 88-89 stand rejected under 35 U.S.C. § 102(b) as anticipated by Uehara et al. ("Uehara").

Applicant respectfully traverses the rejection. Contrary to the Examiner's assertion, Uehara does not anticipate the Applicant's claimed method for the following reasons:

1) Uehara does not overproduce a single target protein in the test cell at all. Rather, Uehara uses a temperature sensitive mutant of Rous Sarcoma Virus, which encodes

multiple proteins, and results in multiple potential phenotypic characteristics, none of which is shown to be a ***responsive change in a phenotypic characteristic*** (*i.e.* a carefully defined phenotypic response as taught by the specification and reiterated in Applicant's previous amendments).

2) Uehara requires a change in temperature in order to elicit any change in a phenotypic characteristic at all. One of ordinary skill in the art would recognize that Uehara's generic phenotypic characteristic is NOT the specific ***phenotypic response*** set forth by the Applicant. To begin with, as a result of (1) above, any generic change exhibited by a "test" cell such as Uehara's would not be principally ascribable to the functioning of the "target" protein $p60^{src}$ under such conditions. As the Examiner is well aware, it is known that the expression levels of hundreds of proteins are profoundly altered when drastic temperature changes are made to the culture conditions of a cell, such as Uehara does.

3) As a result of (1) and (2), Uehara has no protein-of-interest (POI) within the meaning of Applicant's invention to which a phenotypic response can be ascribed. $p60^{src}$ cannot be considered to be a POI according to Applicant's method, because there is no identification of a phenotypic response as set forth by the Applicant that can be ascribed to $p60^{src}$. Uehara's "test" cell and "control" cell are in fact the same cell - ***both*** produce essentially the same level of the POI (as well as the other proteins encoded by the RSV), but are cultured at different temperatures such that profound changes in gene expression, levels of other proteins, and enzyme activities also result.

4) Accordingly, Uehara has not defined a ***responsive change in a phenotypic characteristic*** (i.e. a ***phenotypic response*** as defined by Applicant) that results from the production of the POI in the test cell and that is other than the level of the POI per se. Indeed, Uehara has no such property for any given POI ($p60^{src}$ or otherwise) for all of the reasons given above. Under the conditions employed, it would be virtually impossible for Uehara to defined a responsive change in a phenotypic characteristic and correlate it with the functioning of any given target protein.

5) Herbimycin does not bind to $p60^{src}$. *In vitro* phosphorylation experiments in the authors own hands demonstrated that "herbimycin has no direct effect on src kinase but

destroys its intracellular environment, resulting in an irreversible alteration of the enzyme leading to loss of catalytic activity. (Uehara, p. 674, col. 2, lines 17-22).

The Examiner has cited Uehara et al., Biochem. Biophys.Res. Comm., 1989, Vol. 163, No. 2, pp. 803-809 as evidence that herbimycin inherently binds directly to $p60^{src}$ and inhibits auto and trans-phosphorylation activities of $p60^{src}$ which are associated with cellular changes associated with transformation. To the contrary, one test described in this reference is performed *in vitro*, which indicates nothing more than the fact that under certain *in vitro* conditions herbimycin *may be* capable of binding to $p60^{src}$ in a test tube. The cellular results simply demonstrate that reducing agents such as glutathion oppose the effects of herbimycin. No further conclusions can be drawn regarding the role of herbimycin in a cell, particularly whether or not herbimycin binds to $p60^{src}$ and inhibits its activity in a cell, and the authors' own original work using their temperature sensitive mutant cellular assay, as stated in the authors' own words, did not identify a compound that interacted with (i.e. could "bind to", exert a "direct effect on") $p60^{src}$. (Uehara et al, 1985, p. 674, ibid.). Thus, Uehara conceded in this original work that the cellular results he obtained did *not* result from the identification of a compound that had a direct effect on $p60^{src}$.

Furthermore, it is well known that a primary activity of herbimycin is binding to the chaperone protein hsp90, thereby preventing the association of hsp90 with substrates such as $p60^{src}$ and numerous other proteins. As a result, the level of $p60^{src}$ in a cell treated with herbimycin is reduced significantly, as are other proteins as well. Hsp90 is only one of the intracellular protein targets of herbimycin. See, for example, Blagosklonny, M.V. (2002) Leukemia; 16:455-462. (Exhibit A). In particular, Blagsklonny states as follows:

> The benzoquinone ansamycins GA and herbimycin A, antibiotics produced by yeast, were initially identified as tyrosine kinase inhibitors. However, as it was determined later, the inhibition of kinases by GA and herbimycin A is indirect. In 1986, it has been shown that benzoquinonoid ansamycins have no direct effect on the Src kinase, but instead 'destroy' the intracellular environment. In other words, GA and herbimycin A are not inhibitors of kinases. They target molecular chaperones: Hsp90 and related Grp94.

Blagosklonny, at p.455, left column, last para. through right column, first para.

As Blagsklonny reveals, herbimycin is not a direct $p60^{src}$ inhibitor at all. Furthermore, herbimycin affects a wide variety of proteins, in part as a result of its interactions with chaperone proteins such as Hsp90. By affecting a wide variety of proteins, herbimycin is also inherently non-specific, which is in complete contrast to the essential teachings of Applicant's method, which is directed toward the identification of specific inhibitors or activators of a given POI (Specification, page 1, lines 15-19; '281 patent, Col. 1, lines 10-12).

While Uehara exemplifies a morphological assay for agents that alter a cellular property, it does not provide an assay that can identify agents that are specific inhibitors or activators of a given target protein. It is respectfully noted that, on this basis, Applicant's invention is distinguished from Uehara's morphological assay in the Specification. (See page 3, line 15 to page 4, line 4; '281 patent, Col. 2, lines 9-33)

In short, Uehara does not anticipate Applicant's claimed invention. Uehara does not identify a POI, does not define a responsive phenotypic characteristic, does not identify a responsive phenotypic characteristic that is maintained when the test compound is withdrawn, and does not identify a specific inhibitor or activator of a POI in a cell. Further, Uehara uses reagents and conditions such as a temperature-sensitive mutant virus together with profound changes in culture conditions that one of skill in the art would consider to be contrary to the invention that is disclosed and claimed. Finally Uehara has no conception of a ***responsive change in a phenotypic characteristic*** of a cell or its use as a tool capable of determining specific inhibitors or activators of a given POI.

For all of the reasons as given above, Applicant contends that Uehara does not anticipate the claimed invention. Withdrawal of the rejection is respectfully requested.

Rejections Under 35 U.S.C. § 112, first paragraph

Claims 33-34, 36-37, 43-50, 59-65, 71-78 and 87-89 stand rejected under 35 U.S.C. § 112, first paragraph for failing to comply with the written description requirement. The Examiner asserts that the specification does not enable one of ordinary skill to practice the invention without undue experimentation. In particular, the Examiner relies on Hsiao as evidence of unpredictability - *i.e.*, that a skilled artisan would not be able to distinguish

between chemical agents which directly interact with the POI vs. those which affect the POI by indirect means without additional undisclosed experimentation.

Applicant respectfully asserts that Hsiao provides no basis for disputing predictability of Applicant's claimed invention, because the work described by Hsiao is significantly different from the instant invention. Hsiao's method lacks key elements of the invention. Most notably, Hsiao does not identify a "graded cellular response" or any other responsive change in a phenotypic characteristic. Further, Hsiao does not identify a POI or an activator or inhibitor of a selected POI. Hsiao simply cannot be compared with Applicant's invention.

Most importantly, Hsiao cannot define a responsive change in a phenotypic characteristic according to the teachings of Applicant's method. As would be well known to one of skill in the art, transfecting a population of cells on a single 10 cm tissue culture dish with a given gene such as an activated c-H-ras oncogene (T24) results in dozens or even hundreds of discrete foci that are different from one another - *i.e.*, the foci develop from discrete transformants that contain widely different levels of intracellular $p21^{ras}$, based upon factors that effect gene expression after gene transfer. Such factors include integration site, copy number and natural heterogeneity in the population of host cells that are transfected. The result is that each of the individual tumor foci have widely different levels of p21 in them. Further, the skilled investigator also knows that in some instances transformed foci will emerge that are completely independent of the activity of the transfected gene or cDNA (in this case the T24 oncogene encoding p21 ras) (See for example Specification, page 10, lines 25-30; '281 patent, Col. 5, lines 40-45).

Hsiao cannot possibly identify a ***responsive change in a phenotypic characteristic*** because no correlation is made between $p21^{ras}$ function and $p21^{ras}$ level. To do so, Hsiao would need to isolate individual cell lines and characterize the level of $p21^{ras}$ in those cell lines. But Hsiao *does not isolate any individual cell lines*, much less select any isolated cell line and characterize the level of p21 present. Even if Hsiao had proceeded to determine the level of p21 in any clonal cell line, there was no correlation of $p21^{ras}$ protein activity with any function or defined phenotypic response of the cell line.[1] Thus, Hsiao fails to actually or

[1] Hsiao's work merely demonstrates that tumor promoting phorbol esters can synergize with an activated ras oncogene to increase the number of *distinct* cells that become cancerous (form foci). No further work is performed on the hundreds of gene transfected foci except to simply count them.

conceptually disclose, much less make use of, a graded cellular response or any other responsive change in a phenotypic characteristic.

Applicant respectfully asserts that a prima facie case of non-enablement has not been made out. Hsiao has not identified a POI, has not identified a test cell, has not identified a responsive change in a phenotypic characteristic evoked by the production of a POI in a cell, and has not determined that any agent is an activator or inhibitor of any POI. Hsiao's work does not conform in any way with Applicant's disclosed invention. Hsiao simply has not, even remotely, come close to Applicant's invention.

The significance of Applicant's invention is that it provides a method by which a substance that directly interacts with and inhibits, or directly interacts with and activates, an enzyme of interest can be selectively identified. As stated in the specification, and emphasized in previous arguments, this is because Applicants test method provides test cells having phenotypic responses that are particularly sensitive to substances that directly interact with and inhibit, or directly interact with and activate the overexpressed protein that evokes the presence of the phenotypic response. Furthermore, the specification provides clear guidance for practicing the invention. For example, an outline for practicing the screening method is set forth in the Specification at page 19, line 3 to page 21, line 14 ('281 patent, Col. 8, ln. 34 to Col. 9, ln. 40). A working example demonstrates the responsive change in a phenotypic characteristic that results from overexpression of protein kinase C (PKC) and a correlation between the level of PKC expression and responsiveness of selected phenotypes to inhibitors or activators of PKC. Further, a working example identifies tamoxifen, previously known only as an anti-estrogen, to be a PKC inhibitor in a cellular system.

Although the scope of the claims is broad, the nature of the invention is broad, concerning a general method for identifying inhibitors or activators that interact directly with a given POI. As the Examiner recognizes, the level of skill in the art is high. Applicant asserts that the specification provides adequate guidance as well as working examples, and that no undue experimentation is necessary to practice the claimed invention. By following the disclosed method, one of ordinary skill in the art can expect to identify substances that are inhibitors or activators of a POI that directly interact with the POI. Applicant has previously directed the Examiner's attention to studies published after Applicant's invention that

demonstrate the advantages of relying on cellular function of a POI in cell-based assays to identify or evaluate compounds that bind directly to a POI. (Amendment dated Oct. 22, 2003, pages 23-27) Moreover, in Applicant's own experience, the disclosed method is useful for identification of inhibitors or activators of a given POI.

Claims 33-34, 36-37, 43-50, 59-65, 71-78, and 87-89 stand rejected under 35 USC § 112, 1st paragraph, as failing to comply with the written description requirement. The Examiner continues to assert that Applicant has not disclosed a method that is capable of determining whether a chemical agent directly interacts with a POI and in doing so serves as an inhibitor or activator of the POI.

As set forth above, Applicant respectfully asserts that the Examiner has no reasonable basis for his statement that the method described in the disclosure does not discriminate between chemical agents which directly or indirectly interact with the POI. In contrast to the state of the art at the time of the invention, Applicant has disclosed a method for identifying those compounds that specifically inhibit or activate a given protein in a cell. As noted above, Applicant has explicitly described a method for determining whether a chemical agent both 1) directly interacts with the POI, and 2) is an inhibitor or activator of the POI. The method set forth by the Applicant, for example at page 19, line 3 to page 21, line 14 of the Specification ('281 patent, Col. 8, ln. 34 to Col. 9, ln. 40), accomplishes both parts of the determination. Further, it is believed that the instant claims succinctly recite that method.

Accordingly, Applicant asserts that the claimed invention is fully enabled and described. Applicant respectfully requests that the instant rejections under 35 U.S.C. § 112, first paragraph be withdrawn.

Rejections Under 35 U.S.C. § 112, second paragraph

Claims 33-34, 36-37, 43-50, 59-65, 71-78, and 87-89 stand rejected under 35 USC § 112, second paragraph, as indefinite because it is unclear if the enzyme (POI) must be maintained in an active form in the cell when bound an inhibitor. Also, it is asserted that the claims are vague in that the methods do not recite a step that refers back to or recapitulates the preamble of the claim.

The rejection regarding recapitulation of the preamble of the claim is believed moot in view of the instant claim amendments.

With regard to maintenance of the level of the POI, Applicant has amended the claims to provide that "the level of the enzyme *activity* is maintained" in the cell. Applicant believes that it would be apparent to one of ordinary skill in the art that within the meaning of the claims, maintaining the level of the enzyme is no different from maintaining the level of the active form of the enzyme. That is, the enzyme activity (absent inhibitor or activator) should be maintained such that upon removal of a direct inhibitor or activator of the enzyme, the test cell will be capable of exhibiting the same phenotypic response as prior to addition of the inhibitor or activator. It is believed that the instant amendment makes this clear.

Accordingly, it is respectfully requested that the instant rejection be withdrawn.

Conclusion

It is believed that this amendment is fully responsive to the Examiner's rejections. In view of the foregoing amendments and remarks, it is firmly believed that the subject claims are in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

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REVIEW

Hsp-90-associated oncoproteins: multiple targets of geldanamycin and its analogs

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Geldanamycin (GA), herbimycin A and radicicol bind heat-shock protein-90 (Hsp90) and destabilize its client proteins including v-Src, Bcr-Abl, Raf-1, ErbB2, some growth factor receptors and steroid receptors. Thus, Hsp90-active agents induce ubiquitination and proteasomal degradation of numerous oncoproteins. Depending on the cellular context, HSP90-active agents cause growth arrest, differentiation and apoptosis, or can prevent apoptosis. HSP-active agents are undergoing clinical trials. Like targets of most chemotherapeutics, Hsp90 is not a cancer-specific protein. By attacking a nonspecific target, HSP-90-active compounds still may preferentially kill certain tumor cells. How can this be achieved? How can therapeutic potentials be exploited? This article starts the discussion.
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Keywords: molecular therapeutics; geldanamycin; oncogenes; heat shock proteins

Introduction

When a half-century ago, anticancer drugs were introduced into clinical practice, their mechanisms of action were not fully elucidated. These chemotherapeutic agents attack DNA, inhibit nucleotide metabolism and supress microtubule function. Yet, conventional chemotherapy can cause remissions and even can cure certain malignancies, such as childhood leukemia and testicular cancer. A conceptual basis for standard chemotherapy was inhibition of cycling and killing of dividing cells.[1] Unrestricted cell cycle is a hallmark of cancer.[2–6] However, the toxicity to normal cells (especially to proliferating cells) limits chemotherapy.[7–9]

By the beginning of a new millennium, numerous molecular targets of mechanism-based anticancer drugs have been identified. These targets include growth factor (GF) receptors, mitogen-activated kinases, cyclin-dependent kinases and anti-apoptotic kinases such as Bcr-Abl and Akt.[10–14] Although some of them are etiologic to cancer, they are not cancer-specific. With a few exeptions (eg Bcr-Abl), these oncoproteins also govern life and proliferation of normal cells. Besides, cancer cells usually acquire multiple genetic alterations.[15] Parallel and redundant signaling pathways can support survival and growth of cancer cells.[6,11,12] Therefore, hitting one target may not be sufficient to kill a cancer cell. It has been suggested that in order to reverse the transformed phenotype, it is desirable to identify an agent capable of affecting multiple targets in signal transduction pathways.[16] The concept of the multi-hit modality is emerging.[11,12]

In light of the multi-hit concept, geldanamycin (GA) and other agents that target heat shock protein-90 (Hsp90) are 'wonder drugs'. The benzoquinone ansamycins GA and herbimycin A, antibiotics produced by yeast, were initially identified as tyrosine kinase inhibitors. However, as it was determined later, the inhibition of kinases by GA and herbimycin A is indirect. In 1986, it has been shown that benzoquinonoid ansamycins have no direct effect on the Src kinase, but instead 'destroy' the intracellular environment.[17] In other words, GA and herbimycin A are not inhibitors of kinases. They target molecular chaperones: Hsp90 and related Grp94.[18]

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Heat shock proteins

By definition, heat induces heat shock proteins (Hsps). Heat shock activates the synthesis of only a few proteins and strongly inhibits the synthesis of most others.[19] Hsp with molecular mass 90 (Hsp90) is an abundant cytosilic protein in bacteria and eukaryotes, with homologues of Grp94 in higher eukaryotes. These two proteins are major targets for Hsp-active drugs.[20] Hsps are also referred to as 'molecular chaperones'. A chaperone protein helps other proteins to avoid misfolding pathways that produce inactive or aggregated states. Hsp90 acts in concert with other chaperones and partners (Hsp70, p23, HOP, p50/Cdc) to provide maturation and folding, as well as traficking and function of their client proteins (c-Raf, ErbB-2, steroid receptors). The complexity of multi-chaperone complexes has been extensively reviewed.[20,21] What is important for clinical applications is that inactivation of Hsp90 results in inappropriate functioning and rapid degradation of chaperone's client proteins. Several protein kinases, including Raf-1, ErbB-2, and Bcr-Abl depend upon the chaperone Hsp90 for proper function and stability.[20–23] The benzoquinone ansamycins GA and herbimycin A and the macrocyclic antifungal antibiotic radicicol bind to Hsp90 and specifically inhibit this chaperone's function, resulting in degradation of HSP90-associated proteins.[24–27]

The proteasome and GA-induced degradation

The ubiquitin (Ub)-proteasome pathway is the major non-lysosomal pathway of proteolysis in human cells and accounts for the degradation of most short-lived proteins. Proteins are usually targeted for proteasome-mediated degradation by the covalent addition of multiple units of the 76 amino acid protein ubiquitin (Ub). Ubiquitinylated proteins are degraded by the 26S proteasome, a large protease complex. Normally, many short-lived proteins, such as cyclins or inhibitors of CDK kinases and wild-type p53, are rapidly degraded by ubiquitin-dependent proteolysis.[28,29]

In contrast, Hsp90 prevents degradation of Hsp90 client proteins. By inactivating chaperone function, Hsp90-active drugs permit degradation of ErbB-2 and receptors of IGF, insulin, and EGF.[30,31] The enhanced degradation of receptors can be prevented by inhibitors of the 20S proteasome.[30] For example, within minutes of exposure to GA, mature ErbB-2

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became polyubiquitinated.[31] One can predict that the inhibition of the proteasome will result in accumulation of ubiquitinated proteins (Figure 1). Indeed, treatment of cells with lactacystin, a proteasome inhibitor, blocked GA-induced degradation of ErbB-2 and enhanced the accumulation of polyubiquitinated ErbB-2. Following GA and lactacystin treatment, a higher molecular weight form of ubiquitin-ErbB-2 conjugates was detected.[31] Similarly, cotreatment with the proteasome inhibitor PS-341 reduced GA-mediated degradation of Bcr-Abl.[32] It should be emphasized that inhibition of the proteasome is extremely toxic for a cell. Due to accumulation of certain proteins, inhibitors of proteasome induce apoptosis in leukemia and many non-leukemia cells.[33–35] GA and PS-341 antagonized each other's toxicity in leukemia cells that were transfected with Bcr-Abl.[32,36]

Mutant p53 and geldanamycin

Normally, wt p53 is rapidly degraded by the proteasome.[28,37] Wt p53 transcriptionally induces Mdm-2, which in turn targets p53 for degradation by the proteasome.[38,39] Mutant p53 does not induce Mdm-2 and, therefore, mutant p53 is not degraded and is highly overexpressed. In brief, loss of function causes stabilization of mutant p53.[40] Ectopic Mdm-2 still targets mutant p53 for degradation.[41]

It has been shown, that GA causes degradation of mutant p53.[42] Inhibition of Hsp90 leads to depletion of mutant p53, but not of wild-type p53 in leukemia, breast and prostate cell lines.[42] GA restores p53 polyubiquitination and degradation of mutant p53 by the proteasome.[43,44] However, the mechanism of destabilization of mutant p53 appears to be different from the mechanism of destabilization of most GA-sensitive proteins. For one, there is no evidence that mature p53 binds Hsp-90. In contrast, Hsp90 participates in the achievement of the mutated conformation of nascent p53.[45] GA stimulates degradation of a newly synthesized protein only. In agreement, a rate of p53 depletion is slower than those for Raf-1 and ErbB2. Interestingly, mechanisms of depletion of mutant p53 and CFTR appears to be similar. Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome.[46] Although Mdm-2 plays a small role in the degradation of mutant p53 caused by GA, the alternative mechanism of degradation of mutant p53 still involves ubiquitination and the proteasome.[44]

GA does not change levels of wt p53 and does not affect induction of wt p53 by DNA damage,[42] but it prevents wt p53 accumulation caused by paclitaxel.[47]

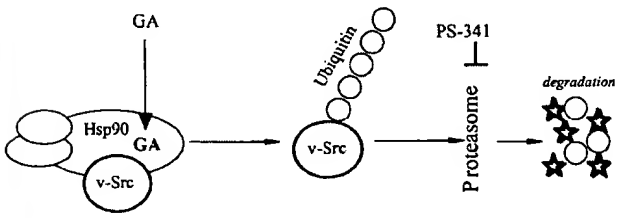

Figure 1 Mechanism of action of Hsp90-active drugs. Geldanamycin (GA) causes degradation of Hsp90-client proteins (eg Src). See text for details.

Mapping the network of oncoprotein's targets

G1/S cell cycle transition comprise a highly nonlinear network from activation of growth factor receptors to cyclin dependent kinases.[3,5] Folding and stability of numerous signaling proteins depend on the Hsp90 function.[20–22] Receptors of GF that are sensitive to GA-mediated degradation include IGF-I, EGF and PDGF receptors, and HER-2.[30,31,48–50] Hsp90-active agents inactivate multiple kinases such as Src, Lyn, Lck, Raf-1 and Cdk-4.[18,51–53] Akt is affected either in a direct[54] or indirect manner (Figure 2). Inhibition of these pathways results in down-regulation of cyclin D1 and functional inactivation of Cdk-4 (Figure 2). In some cell types, cyclin D expression is dependent upon PI3-kinase and Akt which in turn are inhibited by Hsp90-active agents.[55]

Cyclins D are growth factor sensors.[56] Growth factors regulate cyclin D1 by several mechanisms. (1) Transcriptional induction of cyclin D1 that is dependent on the Ras/Raf-1/Mek/ERK pathway.[5] (2) Stabilization and accumulation of the cyclin D protein. In the absence of growth factor signaling, cyclin D1 is rapidly degraded by the proteasome. The pathway that sequentially involves Ras/PI-3 kinase/Akt prevents degradation of cyclin D1. (3) Translocation of cyclin D to the nucleus and its assembly with CDK-4 and CDK-6.[5] All these pathways are blocked by Hsp90-active drugs (Figure 2). In addition, Hsp90-active agents destibilize CDK-4.[53]

Like GA and its derivites, radicicol (a macrocyclic antifungal antibiotic) suppresses transformation caused by Src, Ras and Mos.[57,58] It has been shown that radicicol can inhibit Ras-induced activation of Erk-2.[59] Levels of Raf-1 are decreased in radicicol-treated cells, whereas levels of Ras and Erk-2 remain unchanged. Therefore, like GA, radicicol disrupts v-Src- and Ras-activated signaling pathways by selectively depleting the Raf kinase.[59,60] As it was discovered later, radicicol binds Hsp90 with consequent dissociation of the Raf/Hsp90 kinase

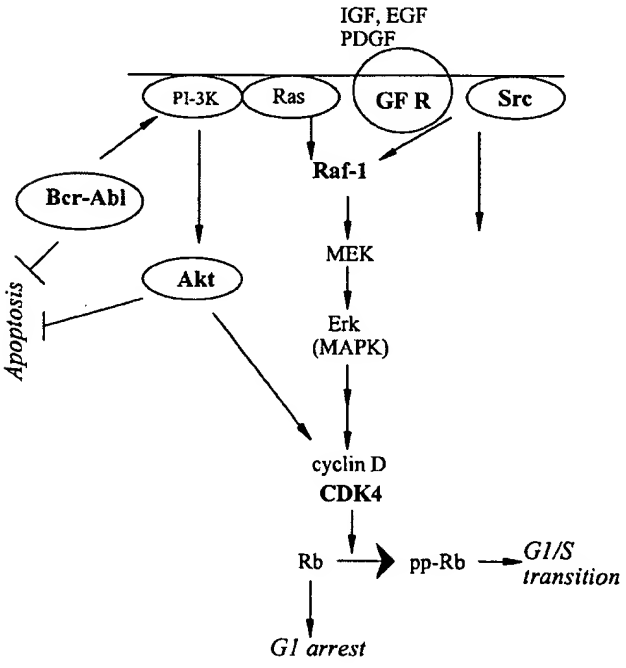

Figure 2 Hsp90-active drugs disrupt proliferative and antiapoptotic signaling pathways. Molecular targets of Hsp90-active drugs are in bold.

complex, leading to the attenuation of the Ras/MAP kinase signal transduction pathway.[61]

Many other experimental therapeutics are aimed at these signaling network: growth factor receptors and tyrosine kinases, Ras, Mek, PI3-K and CDK.[62,63] However, downstream and/or parallel signaling pathways may render cancer cells resistant to growth inhibition. Hsp90-active agents destabilize multiple signaling oncoproteins (Figure 2). By blocking the upstream signaling, GA and herbimycin A inactivate non-target proteins such as Erk1/2.[32,51,64] Although Hsp90-active drugs do not directly target Ras, they block its upstream and downstream signaling pathways. By blocking the Raf-1/MEK pathway, GA abrogated phorbol ester-induced p21 in SKBr3 breast cancer cells[65] and in HL60 leukemia cells.[66]

The following example illustrates the importance of targeting parallel pathways. Elevated levels of urokinase plasminogen activator-1 (uPA) and the IGF-I receptor are associated with breast cancer recurrence and decreased survival. IGF-I requires both PI-3K- and MEK-dependent pathways to optimally induce uPA expression. The production of uPA induced by IGF-I was blocked up to 90% by herbimycin A, but was blocked less potently by LY294002 (an inhibitor of PI-3K) or PD98059 (an inhibitor of MEK).[64]

All Hsp90-active drugs inhibit the same signaling pathways. For example, the designed small molecule PU3, which competes with GA for Hsp90 binding, induces degradation of proteins, including HER-2, in a manner similar to GA. Furthermore, PU3 inhibits the growth of breast cancer cells causing Rb hypophosphorylation, G1 arrest and differentiation.[67]

Glucocorticoid receptors

Activities of steroid hormones are mediated by the superfamily of nuclear receptors, which include those for steroid and thyroid hormones and retinoids. These receptors are ligand-dependent transcription factors that can stimulate gene expression and regulate proliferation, differentiation, and specific functions of target tissues. Unligated receptors exist in inactive complexes with chaperone proteins such as heat-shock proteins (HSPs).[68]

The unliganded glucocorticoid receptor is a complex of a ligand-binding protein, HSP90, HSP70, HSP40, p23 and HOP.[69] Upon binding of glucocorticoids to their receptor, the complex moves along the microtubules to the nucleus. GA disrupts glucocorticoid receptor function.[70] GA impedes hormone-dependent GR translocation along microtubules.[71,72] A functional antagonism between Hsp90-active agents and glucocorticoids should be taken into account in the therapy of leukemia.

Estrogen and progesterone receptors

Estrogens and progestins control cell proliferation of mammary epithelium. Therefore, anti-estrogens is a tissue-specific therapy in breast cancer. Unstimulated estrogen and progesterone receptors exist as multimolecular complexes consisting of the hormone-binding protein itself and several essential molecular chaperones including Hsp90. Hsp90-active drugs (geldanamycin and radicicol) destabilize these hormone receptors in breast cancer cells.[73] *In vivo*, administration of 17-allylaminogeldanamycin (17-A-GA) to estrogen-supplemented, tumor-bearing mice resulted in marked depletion of progesterone receptor levels in both uterus and tumor. It also delayed the growth of hormone-responsive MCF-7 and T47D human tumor xenografts for up to 3 weeks after the initiation of therapy, suggesting that GA can be used in refractory breast cancer.[73]

Mechanisms of growth inhibition

The mechanism of cytotoxicity caused by GA is the degradation of Hsp90-associated proteins. There is a perfect correlation between down-regulation of Hsp90's client proteins and growth inhibition caused by analogs of GA. A near-maximal depletion of Raf-1, ErbB2 and mutant p53 is accompanied by near-maximally toxicity to SKBr3 breast cancer cells.[16] For GA, these concentrations (IC_{80-90} = 30 nM) are between four and five times greater than an IC_{50} (below 10 nM).[16] Similarly, 30 nM GA depleted Bcr-Abl in K562 cells. ErbB-2, Raf-1, Cdk-4 and mutant p53 were depleted by GA analogs and KF25706 (a radicicol oxime derivative) at concentrations comparable to those required for the antiproliferative activity.[16,60] Depletion of the Bcr-Abl protein (at concentrations of GA as low as 30 nM) selectively induced apoptosis in Bcr-Abl positive cells and sensitized these cells to standard chemotherapy.[36]

Hsp90-active drugs induce both G1 and G2/M phase arrest of the cell cycle. Herbimycin A down-regulates cyclin D, causing an Rb-dependent growth arrest in the G1 phase of the cell cycle.[55,74,75] In breast cancer cells, a G1 arrest was accompanied by differentiation and followed by apoptosis. The differentiation was characterized by specific changes in morphology and induction of milk fat proteins and lipid droplets. In cells lacking Rb, neither G1 arrest nor differentiation occurs. Instead, they undergo apoptosis during mitosis.[76]

In K562 leukemia cells, GA induces both G1 and G2/M arrests.[36,77] In these cells, GA down-regulated the expression of cyclin B1 and inhibited phosphorylation of p34Cdc2, causing G2/M arrest.[78] Effects of GA are cell-type dependent. By arresting MCF-7 cells, GA prevents paclitaxel-induced mitotic arrest and Bcl-2 phosphorylation in MCF-7 cells,[79] but not in HL60 cells.[80] Therefore, GA can either decrease or increase the cytotoxicity of paclitaxel, depending on cellular context, that potentially could be exploited therapeutically. Both sequence of drugs and tumor cell biology matters in combining cytotoxics with Hsp90-active drugs.[81] For example, in a subset of breast cancer cell lines, addition of 17-A-GA to cells after exposure to paclitaxel increased apoptosis. In breast cancer cells with intact Rb, such as SKBr3 cells, exposure to 17-A-GA before paclitaxel resulted in growth arrest and abrogated apoptosis.[82] Such a schedule dependence was not seen in BT-549 and MDA-468 breast cancer cells with mutated Rb. Exposure to 17-A-GA before paclitaxel rendered lung cancer cells with low ErbB-2 levels refractory to paclitaxel cytotoxicity.[83] 17-A-GA sensitized breast cancer cells to doxorubicin, in a schedule- and Rb-independent manner.[82] In HL60 leukemia cells, Hsp90-active agents diminished the cytotoxicity of doxorubicin.[36]

In a cell-type dependent manner, Hsp90-active agents cause apoptosis. For example, 17-A-GA induces cytosolic accumulation of cytochrome C, activates caspase-9 and caspase-3, triggering apoptosis in HL-60/Bcr-Abl and K562 cells.[32]

Crucial targets of Hsp90-active drugs vary in different cell types. For example, in Bcr-Abl-expressing K562 leukemia cells, Bcr-Abl appears to be the crucial target which depletion causes cytotoxicity. ErbB-2 is likely an important target in

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SKBr3 breast cancer cells, which overexpress ErbB-2. Context-dependent effects of GA is one of the basis for cancer-specific cytotoxicity.

Bcr-Abl-expressing leukemia

Chronic myelogenous leukemia (CML) is characterized by a reciprocal (t9;22) chromosomal translocation, known as the Philadelphia chromosome, that fuses the truncated *Bcr* gene to the truncated *c-Abl*.[84] Bcr-Abl is an active tyrosine protein kinase, which renders cells resistant to apoptosis.[85–88] Bcr-Abl exists in a complex with Hsp90, and GA causes degradation of Bcr-Abl after 3–5 h of treatment.[77,89] GA down-regulates Bcr-Abl in natural Ph^1-positive K562 cells and in HL60 cells transfected with Bcr-Abl.[32,36,89] In both cell lines, a depletion of the Bcr-Abl protein and a near-maximal toxicity were achieved at concentrations of GA as low as 30 nM.[36] Furthermore, GA sensitized Bcr-Abl-expressing cells to doxorubicin and, albeit to a lesser degree, to paclitaxel.[36]

Specific inhibitors of the Abl kinase, such as STI 571, are very effective in the therapy of Bcr-Abl-positive leukemia,[90,91] but resistance to STI 571 develops.[91–93] Hsp90-active drugs, especially in combination with doxorubicin or STI571, may be a second-line therapy of Bcr-Abl-positive leukemias.

FLT3-expressing leukemias

A somatic mutation of the FLT3 gene, in which the juxtamembrane domain has an internal tandem duplication, is found in 20% of human acute myeloid leukemias. Transfection of mutant FLT3 gene into an IL3-dependent murine cell line, 32D, abrogated the IL3-dependency, and caused leukemia in addition to subcutaneous tumors in mice.[94] Herbimycin A, a Hsp90-active agent, inhibited the growth of the transformed 32D cells, but it was ineffective in parental 32D cells. Herbimycin A suppressed the constitutive tyrosine phosphorylation of the mutant FLT3, but not the phosphorylation of the ligand-stimulated wild-type FLT3.[95] In mice transplanted with the transformed 32D cells, the administration of herbimycin A prolonged the latency of disease or completely prevented leukemia, depending on the number of cells inoculated and schedule of drug administration. These results suggest that mutant FLT3 is a promising target for Hsp90-active drugs in the treatment of leukemia.[95]

Selectivity against cancer cells

Two previous examples illustrate the basis of selective killing of ceratin oncoprotein-expressing cells. In addition to Bcr-Abl and FLT3, Raf-1 may be an important target in leukemias.[63] As shown in Bcr-Abl-expressing cells, acute destabilization of the kinase results in cell death. In breast cancer, simultaneous targeting of ErbB-2, the IGF receptor, the Akt kinase, and estrogen receptors by Hsp90-active drugs may be effective. If a cell particularly depends on a certain oncoprotein, it may fall apart following a brief exposure to Hsp90-active drugs.

Secondly, Hsp90 is overexpressed in tumor cells,[96] indicating that these cells are highly dependent on the Hsp90 function. Mutant oncoproteins may depend on the full function of Hsp90 as a conformational buffer to maintain full activity. Overloading of the Hsp90 capacity with mutated oncoproteins under treatment with Hsp90-active agents could cause death of cancer cells. For example, maintenance of wild-type Hck (a Src-family kinase) and its constitutively active counterpart, Hck499F, requires Hsp90. Hck499F had a greater requirement for on-going support from Hsp90 than did mature wild-type Hck.[97]

Thirdly, Hsp90-active agents can selectively sensitize oncoprotein-overexpressing cells to chemotherapy. Overexpression of ErbB-2 contributes to chemoresistance. 17-A-GA treatment efficiently depleted ErbB-2 in lung cancer cells. Induction of apoptosis was observed after treatment of cells with the combination of paclitaxel and 17-A-GA.[83] Furthermore, Hsp90-active agents can protect certain cells against chemotherapy. This could be exploited for selective killing of cancer cells.

Besides, anti-HER-2 monoclonal antibody can be used to deliver GA, which depletes HER-2, by coupling GA to these monoclonal antibodies.[98] This might enhance the capacity of the antibody to down-regulate HER-2 and also to avoid side-effects of GA.[99]

In a special case, cancer cells that overexpress quinone-metabolizing enzyme (NQO1), which increases in 17-A-GA growth-inhibition activity, can be more sensitive to this HSP90-active compound.[100]

Protection against chemotherapy-induced apoptosis

Hsp90-active drugs can protect some cells against apoptosis caused by other anticancer drugs. For example, cultured dorsal root ganglion (DRG) neurons from chick embryos were extremely susceptible to the antineoplastic drugs, cisplatin, vincristine and paclitaxel even in the presence of the neurotrophins.[101] The neurotoxic effects of these anticancer drugs were completely prevented by the addition of low doses of radicicol (20 nM) or GA (2 nM), but higher doses of GA (>5 nM) had severe cytotoxic effects on neurons. Higher doses of radicicol (500 nM), however, still promoted neurites and prevented apoptosis in the absence of neurotrophins. Slightly different cellular effects of the two antibiotics are not explained.

While potentiating doxorubicin-mediated cytotoxicity in Bcr-Abl-expressing cells, GA protected parental HL60 cells against doxorubicin-induced apoptosis. Combinations of doxorubicin with low concentrations of GA might eliminate Ph-positive cells, while protecting cells that do not express Bcr-Abl. In theory, this can decrease side-effects and increase the therapeutic index.

How can Hsp90-active drugs protect cells from doxorubicin-induced apoptosis? GA and herbimycin A, Hsp90 inhibitors, induce synthesis of Hsp70 and Hsp90.[102,103] In turn, HSPs can inhibit apoptosis.[20] Induction of Hsp70 by herbimycin A was observed in several cell lines, including A431 human epidermoid carcinoma cells, HeLa S3 cells, chicken embryo fibroblasts, NIH3T3 cells and Rous sarcoma virus-transformed NIH3T3 cells.[104] In cardiac cells, herbimycin A induces Hsp70. Moreover, Hsp's induction correlated with the ability of herbimycin A to protect cells against severe stress. These results indicate the possibility of a pharmacological approach to Hsp70 induction and cardiac protection, which may ultimately be of clinical relevance.[105]

Effective concentrations *in vivo*

Concentrations of Hsp90-active drugs that deplete Hsp90-associated oncoproteins can be achieved and tolerated *in*

vivo. Given that GA displayed hepatotoxicity in dogs which appears to be unrelated to its Hsp90 antagonism, 17-allylamino,17-demethoxygeldanamycin (17-A-GA), a geldanamycin analog, is the first inhibitor of Hsp90 that enters a phase I clinical trial in cancer.[81] In mice, bolus i.v. delivery of 60 mg/kg 17-A-GA produced 'peak' plasma 17-A-GA concentrations between 5.8 and 19.3 μg/ml 5 min after injection. After i.v. bolus delivery to mice, 17-A-GA distributed rapidly to all tissues, except the brain. Substantial concentrations of 17-A-GA were measured in each tissue. A 60 mg/kg dose of 17-A-GA, caused no changes in appearance, appetite, waste elimination, or survival of treated animals.[106]

Phase I clinical trials

Although few data are available, one can certainly expect that Hsp90-active agents should be toxic to normal cells. It has been shown, for example, that by inhibiting the Raf-1/MAPK pathway, GA induced apoptosis in luteinized granulosa cells.[107]

In clinical trials, one can retrospectively analyze side-effects due to toxicity to normal cells, thus revealing dose-limiting targets in proliferating (eg mucosa and bone marrow cells) and non-proliferating normal cells. This in turn can help to design rational therapeutic modalities. In a clinical trial at the National Cancer Institute (NIH), 17-allylamino,17-demethoxygeldanamycin (17-A-GA) was administered daily by 1-h infusion for 5 days every 3 weeks in adult patients with various solid tumors.[108] Dose-limiting toxicity was reversible grade III hepatotoxicity. Other grade I/II toxicities included fever, emesis, anemia and fatigue. The maximum tolerated dose was 40 mg/m^2 and plasma maximal concentrations levels were 1860 ± 660 nM. The recommended 17-A-GA phase II dose on this schedule is 40 mg/m^2, at which inhibition of the target Hsp90 occurs.[108] In the clinical trial at Memorial Sloan Kettering in New York, the drug was administered daily for 5 days and repeated every 3 weeks. At 80 mg/m^2 dose (with peak plasma levels of the drug: 2700 nM), limiting toxicities were diarrhea, thrombocytopenia and transient transaminitis.[109] This study suggests that 17-AAG can be administered to patients in concentrations exceeding those that were effective in pre-clinical models.[109] In a phase I trial at Royal Marsden Hospital (UK), with weekly administrations at doses of 80 mg/m^2, no hematological or biochemical toxicity has been observed. In this study, the evidence of biological activity has been demonstrated by induction of Hsp70 in PBLs at 6 h. In the data available by 2001, no objective responses were seen,[109,110] however four of 13 patients had stable disease beyond 3 months.[109]

Two conclusions could be drawn. Firstly, a Hsp90-active drug is not a 'magic bullet' against cancer. Secondly, by shortening the duration of a treatment (1 day instead of 5 days), one may eliminate side-effects. This could be expected. Unlike DNA-damaging drugs, Hsp90-active drugs do not cause irreversible damage, if the time of exposure is brief. Therefore, cells that have not undergone apoptosis potentially may recover.

Although Hsp90-active drugs are introduced in the therapy of solid tumors, preclinical data indicate great promise for these drugs in therapy of leukemia. In striking symmetry, STI571, which was designed to treat Bcr-Abl-expressing leukemia, is underway to explore its utility in solid tumors harboring c-kit and PDGFR abnormalities.

Unexpected twist: instead of conclusion

Thus, Hsp90-active drugs entered clinical trials. But what if Hsp90-active drugs had already been used in therapy. Would it have been a disappointment? Or a useful lesson?

The drug novobiocin has clinically been used for a decade. Coumarin antibiotics, including novobiocin, are known as inhibitors of topoisomerase II. In addition, novobiocin is used clinically as a modulator of alkylating agents.[111] Besides, novobiocin reverses drug resistance and increases intracellular accumulation of etoposide.[112]

Novobiocin (300 μM) induces granulocytic differentiation in HL-60 cells.[113] Finally, at doses of 300–800 μM, novobiocin depleted cells of Raf-1, ErbB2, mutant p53 and v-Src.[114] Although novobiocin binds to a site on Hsp90 that is different from the geldanamycin-binding site, it, like GA, is able to inhibit the chaperone function of Hsp90 and to deplete tumor cells of Hsp90-dependent proteins.[114] Novobiocin therapy decreased levels of Raf-1 in murine splenocytes, indicating that mechanism-based concentration is achievable.[114] Following administration of novobiocin to patients, the serum drug concentration vary between 100 and 400 μg/ml,[115] which corresponds to doses that down-regulate Hsp90-associated proteins *in vitro*.

Thus, the Hsp90-active agent novobiocin is already used in cancer therapy. Side-effects and maximum tolerated dose of novobiocin are difficult to evaluate because the drug was administered in combination with other cytostatics.[111] Given that the severity of mucositis correlated with the plasma levels of novobiocin,[111] one can conclude that novobiocin inhibits proliferation of normal epithelial cells.

It was believed that inhibitors of DNA repair (eg novobiocin) can overcome resistance to alkylators which damage DNA.[111] Ironically, inhibition of DNA repair facilitates development of resistance to alkylating agents.[116] It is tempting to suggest that, by inhibiting Hsp90 functions (rather than DNA repair), novobiocin sensitizes certain tumors to chemotherapy.

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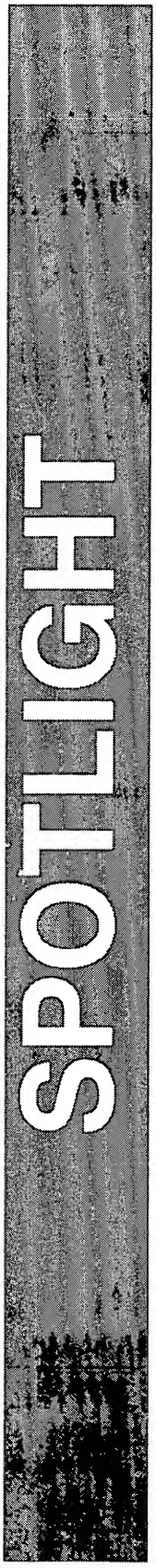

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